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Alternative ejaculate allocation tactics in relation to male mating history of the swallowtail butterfly, *Papilio xuthus* L. (Lepidoptera: Papilionidae)

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Abstract. In polyandrous species, a male's fertilization success is strongly related to the number of sperm he carries and the mass of the ejaculate substance transferred to the female. However, because ejaculate production is costly and limited, males are expected to allocate their ejaculates adaptively among matings. In order to clarify the ejaculate allocation pattern in the polyandrous swallowtail butterfly, *Papilio xuthus* (Linnaeus, 1767), the spermatophore size and the number of sperm were counted just after the termination of the first and second copulations. Virgin males slowly increased the size of their spermatophores with age after eclosion, while there was a negative correlation between the ratio of sperm transferred to the female and the number of sperm produced. Males seemed to keep some sperm for further matings. On the other hand, the spermatophore size rapidly increased in males that had mated once, and these males transferred most of the sperm in their sperm storage organs at their second mating, irrespective of the number of sperm stored. Therefore, males might use their own mating history to tailor their ejaculates, probably assessing the probability of additional matings.

Keywords: apyrene sperm, eupyrene sperm, sperm competition, sperm transfer, spermatophore.

Introduction

When a female insect receives and stores sperm from several males throughout her life, sperm competition commonly occurs in her reproductive organs (Parker, 1970). It has been shown that a male's fertilization success is strongly related to the number of sperm he carries as well as the mass of the ejaculate transferred to the female (Simmons, 2001). In the Lepidoptera, males transfer a single spermatophore that includes two types of sperm, viz. eupyrene and apyrene sperm, in each mating. Various nutrients contained in a spermatophore have been shown to contribute to somatic maintenance in the female (Boggs & Gilbert, 1979) and to increase her fecundity (Watanabe, 1988), and the spermatophore size may affect the length of the refractory period of the female (i.e., the amount of time the female is unreceptive to male courtship following copulation: Sugawara, 1979). On the other hand, Parker (1982) pointed out that a male's fertilization success is often proportional to its contribution to the total number of fertile eupyrene sperm in the female sperm storage organ. In contrast, apyrene sperm is non-fertile, and its role is not yet well known, though apyrene sperm in the female sperm storage organ, the spermatheca, was found to affect the length of the female refractory period relative to a subsequent mating in pierid butterflies (Cook & Wedell, 1999). Therefore, lepidopteran males who can transfer a larger spermatophore with more sperm generally have higher fertilization success, especially in polyandrous species (e.g. Wedell & Cook, 1998).

Sperm production must incur a cost for males (e.g. Dewsbury, 1982). Moreover, in some species, males require time to replenish their ejaculates following copulation, and subsequent copulations often last for much longer (e.g. Svärd & Wiklund. 1989), indicating that the production of other ejaculate substances to be included in the spermatophore is also costly. Thus, in order to maximize their reproductive success, males in polyandrous species should allocate ejaculate resources adaptively among their matings.

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It has been suggested that lepidopteran males can decide how many sperm to transfer in relation to the quality of their mates (e.g. Wedell & Cook, 1999a). For example, male *Plodia interpunctella* (Hübner, 1813) transferred more sperm when mating with more fecund females (Gage, 1998). In some species, males transferred more sperm to mated females (e.g. Solensky & Oberhauser, 2009). On the other hand, condition of the male itself may also affect ejaculate quality. Males who had waited longer until mating transferred larger spermatophores containing more sperm (e.g. Oberhauser, 1988; Watanabe & Hirota, 1999). In addition, males might increase investment into the current mating when the probability of future matings is low (Clutton-Brock, 1984). Wedell & Cook (1999b) stated that the probability of future matings of a male must decrease with the number of matings he has already achieved.

The aim of the present study is to clarify how male butterflies allocate their ejaculate substances in relation to their own mating status in the polyandrous swallowtail butterfly, *Papilio xuthus* (Linnaeus, 1767), in which females mate on average three times during their lifetime (Watanabe & Nozato, 1986). Due to the amount of spermatophore material and because the number of both types of sperm in the males' reproductive organs increase with days since eclosion (e.g. Riemann *et al.*, 1974; Wedell & Cook, 1999b), we first evaluate the production rate of the spermatophore material and both types of sperm in both virgin and previously mated males. To achieve that, we dissected both males and females soon after copula termination. During mating, males do not always transfer all sperm stored. So we dissected males and females just after the termination of copulation to calculate the real number of both types of sperm produced. Thereafter, by using these data, we examined the relationship between the number of sperm transferred to the female and the number of sperm produced until the mating (i.e. sum of sperm transferred plus the sperm remaining in the male reproductive organs) to assess the effect of a male's total actual sperm reserve on the ratio of sperm transferred.

Materials and methods

Mating experiments

In 2008, laboratory-reared adults of *P. xuthus* from the summer generations were weighed on the day of eclosion and given an individual number with a felt-tipped pen on their left ventral hindwing. The sexes were kept separately in flight cages (400×400×450 mm). They were fed on 20% sucrose solution for 10min each day until the first mating.

Virgin males of *P. xuthus* (n=29, one to five days old) were hand-paired with virgin females (one to three days old), as described in detail by Watanabe & Hirota (1999). Once they had copulated, each pair was put into a small cage, and the copula duration was measured. Immediately after the termination of copulation, the females were killed by decapitation and dissected so that the spermatophore mass and the numbers of both types of sperm in the spermatophore could be measured. The males were also killed by decapitation and dissected so that the numbers of both types of sperm that remained in their reproductive organs could be counted. Accordingly, the total number of sperm produced and the rates of transfer of both types of sperm could also be calculated.

To obtain the mated males in the experiment, virgin males (n=16, one day old) were first mated by hand-pairing and then kept in the flight cages until the second mating was induced. They were fed 20% sucrose solution for 10min each day until the second mating. One to five days after the first mating, they were remated with virgin females (one to three days old), again using the hand-pairing method. When the copulation had been terminated by the animals, the male and female were both killed and dissected to measure the spermatophore mass or the number of sperm in the reproductive organs. According to the preliminary observations, because few sperm was found in the duplexes of one-day-old males just after termination of the first copulation, most of the sperm transferred during the second mating that remained in the duplexes of males immediately after the second mating must have been produced later than the first mating.

Dissection of females to remove the spermatophore was started within 20min after the termination of copulation. During this time, no sperm migration from the spermatophore to the spermatheca occurred (Watanabe *et al.*, 2000). The bursa copulatrix was opened, and the intact spermatophore was carefully removed and weighed to the nearest 0.01mg. The numbers of both the eupyrene sperm bundles and free apyrene spermatozoa in the spermatophore were counted. The duplex and vas deferens of each male were also dissected, and the numbers of both eupyrene sperm bundles and apyrene spermatozoa were counted.

Sperm counting procedure

In the Lepidoptera, eupyrene sperm bundles uniformly contain 256 free eupyrene spermatozoa (Virkki, 1969). They start to unravel in the spermatophore only after the termination of copulation, whereas apyrene sperm bundles unravel in the male as soon as they are released from the

testes (Katsuno, 1977). Eupyrene sperm bundles are clearly visible under the stereoscopic microscope at 40× magnification and appear uniform in size (Fig. 1a). Therefore, we mechanically disrupted the spermatophore, duplex and vas deferens, and counted the number of eupyrene sperm bundles directly under a stereoscopic microscope. Then, the ejaculates from each reproductive organ were washed in a small tube containing a known volume of saline water (Ringer's solution for insects). The tube was gently stirred for 1min to homogenize the spermatozoa suspension. A total of six subsamples (10μl) were removed from each primary sample using an autopipette and were allowed to dry on slides under dust covers. The dry slides were dipped in distilled water for approximately 3s and were then allowed to dry again. Each subsample was examined under dark-field phase-contrast microscopy at 100× magnification to count the number of apyrene spermatozoa (Fig. 1b). The number of apyrene spermatozoa in the spermatophore, duplex and vas deferens was calculated by multiplying the average 10μl sperm count by its dilution factor.

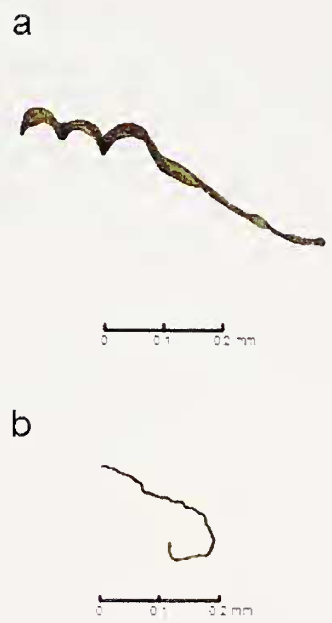

Figure 1. A photograph of an eupyrene sperm bundle (a) and an apyrene spermatozoon (b) from a spermatophore of *Papilio xuthus* under the stereo-microscope.

Statistical analyses

Analyses were mainly performed using SPSS 12.0J for Windows. The copula duration of mated males was compared with that of virgin males in each resting period by using the Mann-Whitney U-test. Data including the spermatophore mass and the numbers of both types of sperm produced (sum of sperm transferred in the spermatophore plus the sperm remaining in the male reproductive organs) were analyzed using ANCOVA, with the male mating history (whether males mated for the first time or had mated previously) as a fixed factor and the number of days until mating (the number of days from eclosion until a male's first mating, or the number of days between a male's first and second mating) as a covariate. The numbers of eupyrene sperm bundles and apyrene spermatozoa transferred in a spermatophore were log-transformed and analyzed using ANCOVA, with male mating history as a fixed factor and the log-transformed numbers of each eupyrene sperm bundles and apyrene spermatozoa produced as covariates. We also tested if the coefficients of the regression lines of the ratio of sperm transferred were different from unity. In log-transformed space, a regression coefficient not significantly different from unity indicates that the ratio of sperm transferred did not change with the number of sperm produced. This analysis was performed using Microsoft Excel 2003. All numerical results are presented as mean±SE.

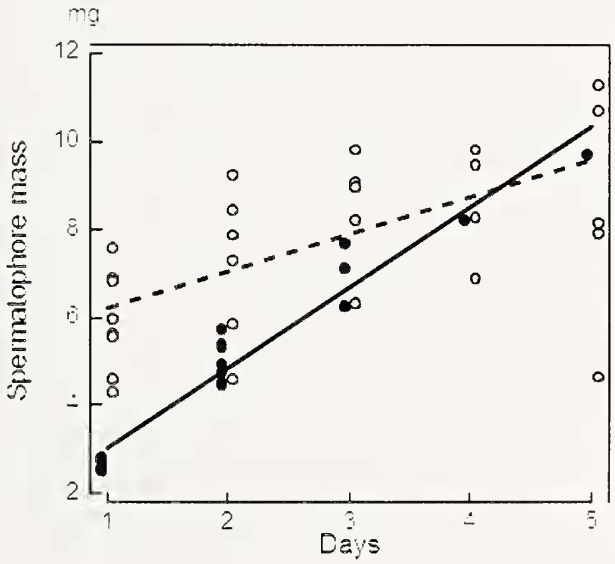

Figure 2. Changes in spermatophore mass produced at the first mating by virgin *Papilio xuthus* males (open circles) relative to days after eclosion, and at the second mating by mated males (solid circles) in relation to the number of days after their first mating. The dotted line represents the virgin males (Y=5.34+0.85X, r^2=0.35, n=29), and the solid line represents the mated males (Y=1.19+1.83X, r^2=0.94, n=16).

Results

The hand-pairing method was effective in allowing each virgin and mated male to copulate successfully. As shown in Table 1 the copula lasted for approximately 1h in virgin males, and there was no significant difference among the days of resting (ANOVA; F=0.534, p=0.71). In previously mated males the copula lasted also approximately 1h, and duration was not significantly different from that of virgin males at each day until the focal mating.

A gel-like accessory gland substance and a teardrop-shaped spermatophore containing eupyrene sperm bundles and free apyrene spermatozoa were observed in the bursa copulatrix of the female in each copulation experiment. Males did not retain spermatophore substance in the simplex, while some eupyrene sperm bundles and free apyrene spermatozoa remained in the duplex and vas deferens.

A one-day-old virgin male on average transferred a spermatophore of 5.9±0.4mg (n=8). The spermatophore mass transferred by virgin males increased with days after eclosion, and five-day-old males produced a spermatophore of 9.4±1.3mg (n=6). On the other hand, mated males one day after the first mating transferred a spermatophore of only 2.7±0.1mg (n=4). The spermatophore mass produced by mated males also increased with resting periods. However, the interaction term between male mating history and days was significant (Table 2), indicating that the increase in the spermatophore mass of mated males was significantly higher than that of virgin males. Spermatophore mass of males five days after the first mating was roughly equal to that of five-day-old virgin males (Fig. 2).

The number of eupyrene sperm bundles produced by a male was the sum of the sperm transferred with the spermatophore plus the sperm remaining in the male reproductive organs. This amounted to approximately 41 eupyrene bundles for one-day-old virgin males, while males mated one day after the first mating produced 34 bundles. While the number of resting days significantly affected the number of eupyrene sperm bundles produced, the interaction term between male mating history and days was not significant (Table 2), indicating that the increase in the number of eupyrene sperm bundles produced was not different between virgin males and mated males. Five days after eclosion or first mating, both the virgin and mated males had produced approximately 150 eupyrene sperm bundles (Fig. 3a).

One-day-old virgin males produced approximately 263,000 apyrene spermatozoa, compared to 163,000 for males re-mated one day after the first mating. While the number of resting days significantly influenced the number of apyrene spermatozoa produced, the interaction term between male mating history and days was again not significant (Table 2), indicating that the increase in the number of apyrene spermatozoa produced was not different between virgin males and mated males. Five days after eclosion or initial mating, the virgin and mated males had produced approximately 650,000 and 500,000 apyrene spermatozoa, respectively (Fig. 3b).

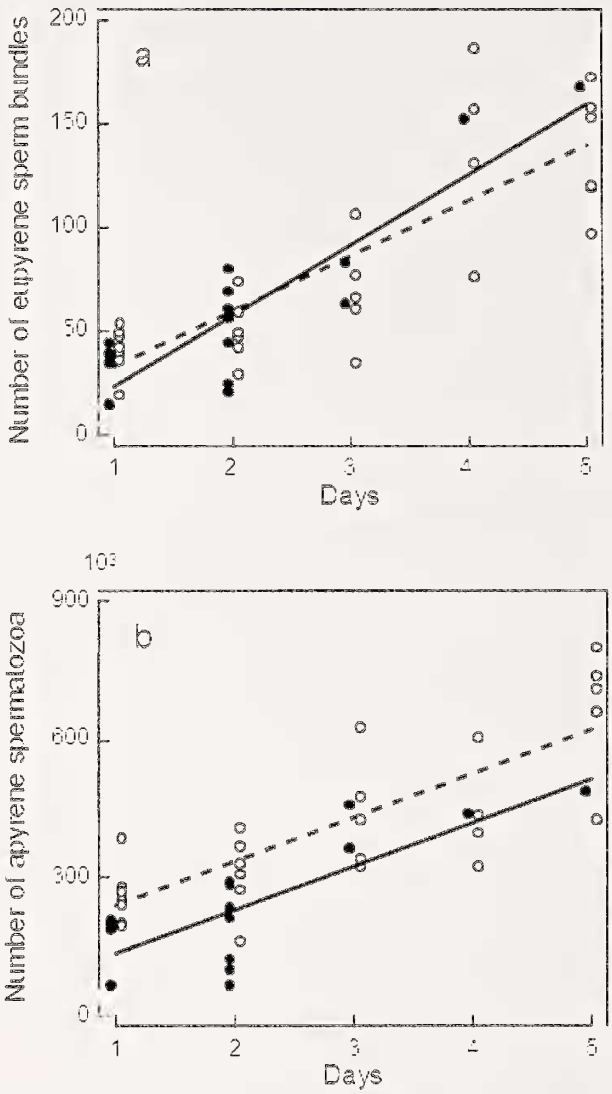

Figure 3. Changes in the number of eupyrene sperm bundles (a) and apyrene spermatozoa (b) produced before the first mating in virgin males (open circles) relative to days after eclosion, and before the second mating in mated males (solid circles) in relation to days after their first mating. Dotted line: virgin males (a) Y=7.01+26.44X, r^2=0.69; (b) Y=144566+95159X, r^2=0.66, n=29); solid line: mated males (a) Y=-11.36+34.16X, r^2=0.79; (b) Y=36150+96052X, r^2=0.63, n=15).

Table 1. Copula duration (min) of virgin males and mated males (mean±SE). For virgin males 'day of resting' means the days since eclosion, while for mated males it means days after the first mating. Figures in parentheses are sample sizes.

Days of resting	1	2	3	4	5
Virgin males	60.8±2.7 (8)	61.7±1.1 (6)	64.8±7.6 (5)	65.3±5.2 (4)	68.0±4.3 (6)
Mated males	57.0±5.0 (4)	60.7±2.0 (7)	57.0±0.6 (3)	59.0 (1)	91.0 (1)
Mann-Whitney U-test	U=14.0, p=0.81	U=18.0, p=0.73	U=6.5, p=0.79	U=1.5, p=0.80	U=0.0, p=0.29

Table 2. Effects of mating history and days until mating on spermatophore mass and number of both types of sperm produced in *P. xuthus*. Given are *F*-values from an analysis of covariance. Degrees of freedom were df = 1;44 for all variables except for spermatophore mass (where df = 1;45). *p<0.05; ***p<0.001.

	Source of variation		
Response variable	**Mating**	**Days**	**Interaction**
Spermatophore mass	15.804***	46.964***	6.177*
Number of eupyrene sperm produced	1.067	80.594***	1.309
Number of apyrene sperm produced	2.447***	52.855***	0.001

Table 3. Effect of mating history and the number of sperm produced on the number of sperm transferred in *P. xuthus*. Given are *F*-values from an analysis of covariance. Degrees of freedom: 1;44 for both response variables. *p<0.05; ***p<0.001.

	Source of variation		
Response variable	**Mating**	**No. sperm produced**	**Interaction**
Number of eupyrene sperm bundles transferred	5.152*	221.820***	5.064*
Number of apyrene spermatozoa transferred	0.516	112.415***	0.404

Out of the 41 eupyrene sperm bundles produced by one-day-old virgin males, approximately 40 (97.6%) were transferred during their first mating, while five-day-old virgin males transferred approximately 95 out of 138 (70.0%) eupyrene sperm bundles produced. The positive relationship between the number of eupyrene sperm bundles produced and the number of eupyrene sperm bundles transferred to the female during copulation indicated that the number of sperm transferred increased with the number of sperm produced (Fig. 4a). However, the regression coefficient was significantly lower than unity (t=-4.102, p<0.001), indicating that the ratio at which eupyrene sperm bundles were transferred by virgin males decreased as the production of eupyrene sperm bundles increased.

For mated males, a positive relationship was also found between the number of eupyrene sperm bundles produced and the number of eupyrene sperm bundles transferred (Fig. 4a). However, the regression coefficient of mated males was not significantly different from unity (t=-0.154, n.s.), indicating that the ratio at which eupyrene sperm bundles were transferred did not change along with the number of eupyrene sperm bundles produced. In addition, the interaction term between male mating history and the number of eupyrene sperm bundles produced was significant (Table 3), indicating that the regression coefficient of mated males was significantly different from that of virgin males.

Out of 263,000 apyrene spermatozoa produced in one-day-old virgin males, approximately 245,000 (94.0%) were transferred in their first mating, while five-day-old virgin males transferred approximately 493,000 out of 665,000 (75.6%) apyrene spermatozoa produced. The number of apyrene spermatozoa transferred increased with the number of apyrene spermatozoa produced (Fig. 4b). The regression coefficient was significantly lower than unity (t=-2.593, p<0.05), indicating that the ratio at which apyrene spermatozoa were transferred by virgin males decreased with the number of apyrene spermatozoa produced.

For mated males, a positive relationship was also found between the number of apyrene spermatozoa produced and the number of apyrene spermatozoa transferred (Fig. 4b). The regression coefficient of mated males was not significantly different from unity (t=-1.064, n.s.), indicating that the ratio at which apyrene spermatozoa were transferred by mated males did not change with the number of apyrene sperm produced. The interaction term between male mating history and the number of apyrene spermatozoa produced was not significant (Table 3).

Discussion

There was a significant increase in spermatophore mass in both virgin males and mated males with the number of days that had passed until the first and the second mating, respectively. For virgin males, spermatophore mass of five-day-old males was 1.5 times as large as that of one-day-old males. The spermatophore from the second copulation (one day after the first copulation) was less than half the size of the first, as reported by Watanabe & Hirota (1999). Spermatophore mass then increased with the duration of delay until the second mating in mated males, but the rate of increase in these mated males was about double that of the virgin males. As a result, spermatophore mass in mated males five days after the first mating was almost as large as that of five-day-old virgin males. Because females that receive a small spermatophore will remate sooner (Kaitala & Wiklund, 1994), males benefit from transferring a large spermatophore at each mating to ensure high reproductive success. Thus, the rapid increase in spermatophore size after the first mating is likely adaptive as it would allow males to engage in frequent mating in polyandrous mating systems. Comparative studies have also shown that spermatophore size in males of more polyandrous species recovered more rapidly (Svärd & Wiklund, 1989; Bissoondath & Wiklund, 1996).

Constancy of the copula duration might be also an adaptation for female polyandry. In the monandrous congener, *P. machaon*, the copula duration increased 9-fold when mated males mated a second time on the day after the first mating (Svärd & Wiklund, 1986). Such a prolonged copulation must incur a time cost for males. To achieve the frequent matings in a polyandrous mating system, males of *P. xuthus* might not increase duration of the copula, even if they have not transferred a sufficiently large spermatophore yet.

Giebultowicz *et al.* (1988) showed that sperm released from the testis to the duplex followed a daily rhythmic pattern, resulting in an increased number of both types of sperm in the duplex with days until

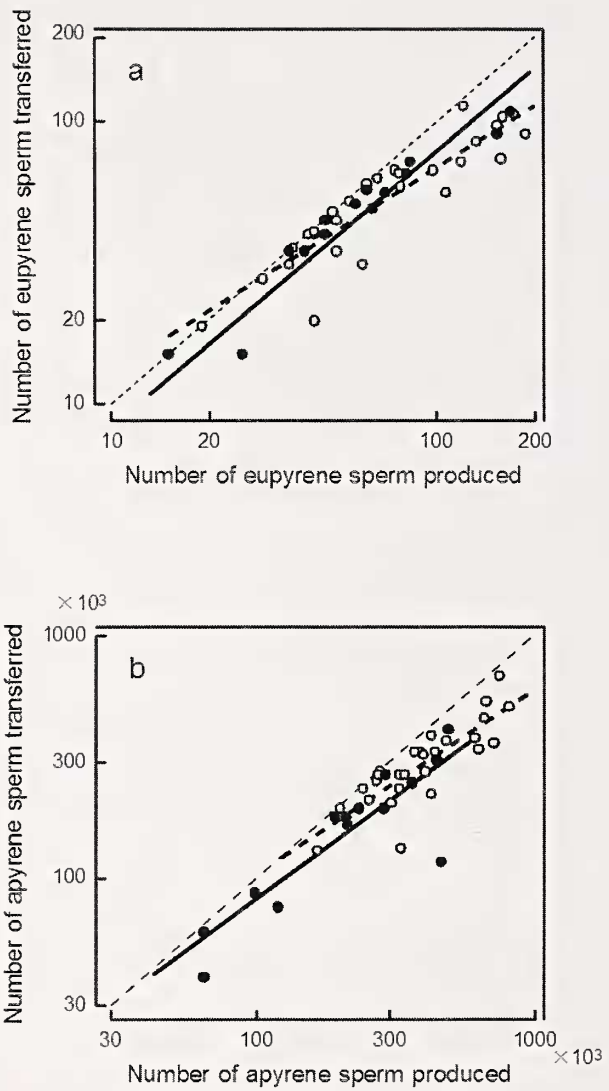

Figure 4. Relationship between the number of eupyrene sperm bundles produced (a) and of apyrene spermatozoa produced (b) to the number of eupyrene sperm bundles transferred. Virgin males: open circles with dotted line; previously mated males: solid circles with solid line. (a) virgin: logY=0.39+0.73logX, r^2=0.82, n=29; mated: logY=-0.07+0.98logX, r^2=0.88, n=15. (b) virgin: logY=1.23+0.76logX, r^2=0.71, n=29; mated males: logY=0.62+0.86logX, r^2=0.76, n=15.

mating. Sperm is likely accumulated in the duplex with age in males (Hiroyoshi & Mitsuhashi, 1999). In the present study, older males that had mated the same number of times stored more sperm than younger ones, which might be beneficial for older males.

Although the number of sperm transferred increased with the number of sperm produced, the sperm transfer ratio was low in virgin males which produced more sperm. An upper limit on the amount

of sperm transfer might explain the decrease in the ratio of sperm transferred with the number of sperm produced. However, the bursa copulatrix of lepidopteran females can expand to include multiple ejaculates (e.g. Drummond, 1984), and sperm is only a small fraction of the ejaculate. Hence, sperm availability is unlikely to act as major limitation to the number of sperm transferred. Rather, virgin males probably regulate the ratio of sperm transferred in relation to their own sperm reserve for future matings. Because the number of sperm produced was associated with male age, we cannot rule out the possibility that the ratio of sperm transferred decreased with male age. But this is unlikely. Clutton-Brock (1984) pointed out that older males should invest more in the current mating than younger males due to the lower probability of future matings, in agreement with the terminal investment hypothesis.

Mated males transferred sperm at a stable ratio irrespective of the number of sperm produced. Most of the sperm produced by mated males was transferred during the current mating. Although the regression coefficient of the number of apyrene spermatozoa transferred on the number of apyrene spermatozoa produced was not significantly different between virgin males and mated males, males seemed to change their sperm ejaculation tactics after the first mating. Because the probability of future matings must be negatively associated with the number of matings already achieved (Wedell & Cook, 1999b), a mated male attains higher reproductive success by transferring as much sperm as possible to his current mate. Therefore, the difference in the sperm ejaculate tactics between virgin and mated males might correspond to the probability of future matings.

Ejaculate allocation might be affected not only by the probability of future matings, but also by other life-history traits. Cook & Wedell (1996) showed that in *Pieris rapae* (Linnaeus, 1758) mated males transferred a smaller spermatophore than virgin males, although larger numbers of both types of sperm were included. This might be an adaptation to the different risks of sperm competition between the first and the second mating (Wedell & Cook, 1999b). In *P. xuthus*, males accelerated the production speed of spermatophore material after their first mating. In addition, the ratio of sperm transfer was higher in the second mating than in the first mating. These results suggest that males need to transfer a large spermatophore with much sperm in the second mating. Due to the long flight season and their relatively long adult lives (Watanabe & Kobayashi, 2006), the age structure of females in the populations is complex. Thus, males could have a high likelihood of encountering females of various levels of fecundity and with various mating histories. Changes in the rate of production or in the ratio of transfer of ejaculate substances might therefore reflect the low predictability of the mating history of males' future mates.

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Literature cited

Bissoondath, C. J. & C. Wiklund. 1996. Male butterfly investment in successive ejaculates in relation to mating system. Behavioral Ecology and Sociobiology 39: 285–292.

Boggs, C. L. & L. E. Gilbert. 1979. Male contribution to egg production in butterflies: evidence for transfer of nutrients at mating. Science 206: 83–84.

Clutton-Brock, T. H. 1984. Reproductive effort and terminal investment in iteroparous animals. American Naturalist 123: 212–229.

Cook, P. A. & N. Wedell. 1996. Ejaculate dynamics in butterflies: a strategy for maximizing fertilization success? Proceedings of Royal Society of London B 263: 1047–1051.

Cook, P. A. & N. Wedell. 1999. Non-fertile sperm delay female remating. Nature 397: 486.

Dewsbury, D. A. 1982. Ejaculate cost and male choice. American Naturalist 119: 601–610.

Drummond, B. A. 1984. Multiple mating and sperm competition in the Lepidoptera, pp.291–370. *In*: Smith R. L. (ed.), Sperm competition and evolution of animal mating systems. Academic Press, London.

Gage, M. J. G. 1998. Influences of sex, size, and symmetry on ejaculate expenditure in a moth. Behavioral Ecology 9: 592–597.

Giebultowicz, J. M., R. A. Bell & R. B. Imberski. 1988. Circadian rhythm of sperm movement in the male reproductive tract of the gypsy moth, *Lymantria dispar*. Journal of Insect Physiology 34: 527–532.

Hiroyoshi, S. & J. Mitsuhashi. 1999. Sperm reflux and its role in multiple mating in males of a butterfly *Polygonia c-aureum* Linnaeus (Lepidoptera: Nymphalidae). Journal of Insect Physiology 45: 107–112.

Kaitala, A. & C. Wiklund. 1994. Polyandrous female butterflies forage for matings. Behavioral Ecology and Sociobiology 35: 385–388.

Katsuno, S. 1977. Studies on eupyrene and apyrene spermatozoa in the silkworm *Bombyx mori* L. (Lepidoptera: Bombycidae) 1. The intratesticular behaviour of the spermatozoa at various stages from the 5th-instar to the adult. Applied Entomology and Zoology 12: 142–153.

Oberhauser, K. S. 1988. Male monarch butterfly spermatophore mass and mating strategies. Animal Behaviour 36: 1384–1388.

Parker, G. A. 1970. Sperm competition and its evolutionary consequences in insects. Biological Reviews 45: 525–567.

Parker, G. A. 1982. Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. Journal of Theoretical Biology 96: 281–294.

Riemann, J. G., B. J. Thorson & R. L. Ruud. 1974. Daily cycle of release of sperm from the testes of the Mediterranean flour moth. Journal of Insect Physiology 20: 195–207.

Simmons L. W. 2001. Sperm competition and its evolutionary consequences in the insects. Princeton University Press, Princeton, New Jersey.

Solensky, M. J. & K. S. Oberhauser. 2009. Male monarch butterflies, *Danaus plexippus,* adjust ejaculates in response to intensity of sperm competition. Animal Behaviour 77: 465–472.

Sugawara, T. 1979. Stretch reception in the bursa copulatrix of the butterfly, *Pieris rapae crucivora,* and its role in behaviour. Journal of Comparative Physiology 130: 191–199

Svärd, L. & C. Wiklund. 1986. Different ejaculate delivery strategies in first versus subsequent matings in the swallowtail butterfly *Papilio machaon* L. Behavioral Ecology and Sociobiology 18: 325–330.

Svärd, L. & C. Wiklund. 1989. Mass and production rate of ejaculates in relation to monandry/polyandry in butterflies. Behavioral Ecology and Sociobiology 24: 395–402.

Virkki, N. 1969. Sperm bundles and phylogenesis. Zeitschrift für Zellforschung 101: 13–27.

Watanabe, M. 1988. Multiple matings increase the fecundity of the yellow swallowtail butterfly, *Papilio xuthus* L., in summer generations. Journal of Insect Behavior 1:17–29.

Watanabe, M., M. Bon'no & A. Hachisuka. 2000. Eupyrene sperm migrates to spermatheca after apyrene sperm in the swallowtail butterfly, *Papilio xuthus* L. (Lepidoptera: Papilionidae). Journal of Ethology 18:91–99.

Watanabe, M. & M. Hirota. 1999. Effects of sucrose intake on spermatophore mass produced by male swallowtail butterfly *Papilio xuthus* L. Zoological Science 16:55–61.

Watanabe, M. & T. Kobayashi. 2006. Total sperm ejaculation in monandrous (*Papilio machaon*) and polyandrous (*P. xuthus*) swallowtail butterflies (Lepidoptera: Papilionidae) restricted to larval stage-derived nutrients. Journal of Research on Lepidoptera 39: 1–7.

Watanabe, M. & K. Nozato. 1986. Fecundity of the yellow swallowtail butterflies, *Papilio xuthus* and *P. machaon hippocrates,* in a wild environment. Zoological Science 3: 509–516.

Wedell, N. & P. A. Cook. 1998. Determinants of paternity in a butterfly. Proceedings of Royal Society London B 265: 625–630.

Wedell, N. & P. A. Cook. 1999a. Butterflies tailor their ejaculate in response to sperm competition risk and intensity. Proceedings of Royal Society London B 266: 1033–1039.

Wedell, N. & P. A. Cook. 1999b. Strategic sperm allocation in the small white butterfly *Pieris rapae* (Lepidoptera: Pieridae). Functional Ecology 13: 85–93.